

CHAPTER ONE

INTRODUCTION

1.1 Introduction:

Skin cancer is now the most common type of cancer in fair skinned population in many parts of the world. The incidence, morbidity and mortality rates of skin cancers are increasing (Deevya, *et al.*2010).

There are three main types of skin cancer includes basal cell cancer (BCC), squamous cell cancer (SCC) and melanoma. The first two together along with a number of less common skin cancers are known as non-melanoma skin cancers (NMSC). Skin cancer is the most common form of cancer, globally accounting for at least 40% of cases. The three main types of skin cancer have become more common in the last 20 to 40 years, especially in those areas which are mostly Caucasian (Cakir, *et al.*2012). Of NMSC about 80% are basal cell cancers and 20% are squamous cell cancers (Sajjad and Jerry, 2008).

Worldwide the incidence for NMSC varies widely with the highest rates in Australia (>1000/100.000 person years for BCC) and the lowest rates in parts of Africa (<1/100.000 person years for BCC). The average incidence rates in England were 76.21/100.000 person years and 22.65/100.000 person years for BCC and SCC respectively (Lomas, *et al.*2012).

In Sudan SCC is the commonest skin malignancy accounting for 42.6% followed by BCC which accounting for 32%. Skin cancer is more common among people with darker white skin and light brown skin (skin photo type III and IV) (Abdelsamei, *et al.*2012).

Exposure to sun light for long times, sun burn, scars, tanning beds, radiation therapy and exposure to arsenic and chemicals, people with fair skin (light skin,

albinism, blue eye), family history of skin cancer, personal history of skin cancer, immune suppressing drugs, smoking, inherited syndrome, chronic inflammation, infections such as HPV increase the risk for developing skin cancer (Saladi and Persaud, 2005).

Histopathologic evaluation of biopsy is the criterion standard for diagnosis of skin cancer (Ashely, *et al.* 2015). Optical diagnostic techniques such as coherence tomography, fluorescence spectrometry, Raman spectroscopy, reflectance spectrometry and confocal microscopy proved to be effective in the diagnosis of skin cancer (Calin, *et al.*2013).

There are several choices for the treatment of skin cancer depend on size, depth, location of the skin cancer and overall health which include excision, curettage and electrodesiccation, cryosurgery, medication, photodynamic therapy, radiotherapy, immunotherapy and chemotherapy (Mark, 2010).

CD10 is a 100-kd transmembrane glycoprotein initially identified as the common acute lymphoblastic leukemia antigen, or CALLA (Dong, *et al.*2003).

CD10 expression exhibits a link with the growth rate of the cells. Its expression is increased in malignant tumors and regenerating tissues, but it is not lineage specific (Wagoner, *et al.*2007).

Within normal adult skin, CD10 immunopositivity has been noted in the inner sheath of hair follicles, hair matrix and perifollicular fibrous sheath (Yada, *et al.*2004).

CD10 is a useful immunohistochemical marker in the differentiation between BCC and SCC, if tumor cells were CD10 positive, this would favor BCC over SCC (Hayam and Hayam, 2007).

1.2. Objectives:

General objectives: To study the value of CD10 in the differentiation between basal cell carcinoma and squamous cell carcinoma.

Specific objectives:

- 1-To detect CD10 in skin cancer tissues using immunohistochemical method.
- 2-To correlate the CD10 expression with histological diagnosis and cancer grade.

CHAPTER TWO

LITERATURE REVIEW

2-Literature review:

2.1 Anatomy and physiology of the skin:

The skin is the largest organ of the body, making up 16% of body weight, with a surface area of 1.8 m. It has several functions, the most important being to form a physical barrier to the environment, allowing and limiting the inward and outward passage of water, electrolytes and various substances while providing protection against micro-organisms, ultraviolet radiation, toxic agents and mechanical insults. In humans skin pigmentation varies among population, and skin type can range from dry to oily, such skin variety provides a rich and diverse habitat for bacteria that number roughly 1000 species from 19 phyla (Elizabeth, *et al.*2009).

The integumentary system is formed by the skin and its derivative structures; the skin is composed of three layers: The epidermis, dermis and subcutaneous layers (Kanitakis,2002).

2.1.1 Epidermis:

It is the outer layer of the skin ranging from 0.1mm to 1.6mm thickness depending on the location on the body (Goldsmith, 1991). It is stratified, squamous epithelium layer that is composed primarily of two types of cells; keratinocytes and dendritic cells (Murphy, 1997).

2.1.2 Dermis:

The dermis is an integrated system of fibrous and amorphous connective tissue that accommodates nerve and vascular network, epidermal derived appendages, fibroblasts, macrophages and mast cells. Elastic and collagen tissue are the main types of fibrous connective tissue. Elastic fibers provide elasticity to the skin while collagen fibers provide tensile strength to the skin (Prost, *et al.*2008).

2.1.3 Subcutaneous layer:

Underlying the dermis is a regionally thick layer of loose, fatty connective tissue; the hypodermis. This layer serves to give flexible attachment of the skin to underlying structures and conducts the vital nerve and blood vascular supply to the dermis (Ronald, *et al.*1996).

The tissue of the hypodermis insulates the body, serves as a reserve energy supply, cushions and protects the skin, and allows for its mobility over underlying structures. It has a cosmetic effect in molding body contours. The boundary between the deep reticular dermis and the hypodermis is an abrupt transition from a predominantly fibrous dermal connective tissue to a primarily subcutaneous one (David, 2012).

2.2 Pathology of the skin:

2.2.1 Inflammatory skin disorders:

Skin inflammation is a physiological reaction to tissue injury, pathogen invasion and irritants. During this process innate and/or adaptive immune cells are activated and recruited to the site of inflammation to either promote or suppress inflammation (Uluckan, *et al.*2015).

Atopic dermatitis and psoriasis are two common chronic inflammatory skin diseases. Both are multifactorial disorders caused by genetic and environmental factors (Rodriguez, *et al.*2011).

Acne is a chronic inflammatory disease of the sebaceous-pilosebaceous unit. Acne development is linked to the combination of predisposing genetic factors and environmental triggers among which a prominent role is played by the follicular colonization by propionic bacterium *acnes* (*P.acnes*) (Antiqua, *et al.*2015).

2.2.2 Precancerous lesion of the skin:

Precancerous skin lesions represent the early stage of skin tumors. There is no invasive tumor growth, so the basement membrane is completely intact. These lesions show a wide variation of clinical and histological appearances on the

skin or mucosa. The precancerous lesion such as actinic keratosis, actinic cheilitis, cutaneous horns, arsenical keratosis, Bowen's disease, intraepithelial neoplasia (Kruger, *et al.*2013).

2.2.3 Malignant tumor of the skin:

2.2.3.1 Squamous cell carcinoma:

SCC is the second most common non melanoma skin cancer. It originates from epidermal keratinocytes or adnexal structures (such as eccrine glands or pilosebaceous units) (Kallini, *et al.*2015).

SCC is twice as common in men as in women. There is an inverse relationship between skin pigmentation and SCC incidence, largely because of protective effect of melanin. Thus, persons with fair skin are at greatest risk (Kricke, *et al.*1990).

In white men and women, the majority of SCC arise on sun exposed areas such as the head, neck and dorsal hands. SCC of the legs is more common in women. In blacks SCC tend to be distributed equally on sun protected and sun exposed areas (Douglas and David, 2012).

Biopsy leads to definitive diagnosis. Treatment includes surgical excision, Mohs micrographic surgery produces excellent cure rates and spares the maximal amount of tissue. Other modalities include electrodesiccation and curettage, cryosurgery, radiotherapy, topical medications, photodynamic therapy and systemic therapy (Kallini, *et al.*2015).

2.2.3.2 Basal cell carcinoma:

BCC is a malignant epithelial neoplasm of the skin preferentially affecting male Caucasians and is rarely observed in patients with more intense skin pigmentation. A characteristic feature of BCC is the low risk to metastasize. The overall incidence is increasing worldwide significantly by about 3-10% per annum (Roewert, *et al.*2007).

BCC is primarily caused by heavy episodic and chronic sun exposure. Predisposing factors include fair skin type, immune suppression and certain

genetic disorders e.g. albinism, Gorlin syndrome, xeroderma pigmentosum (Reinauer, *et al.*2014).

BCC affects mainly photoexposed areas, in about 80% of patients it appears in the head and the other photoexposed areas such as the trunk and the limbs are less affected, in about 4% of patients may appear on genitals and perianal area. The tumor has slow progression and metastases are found in only 0.5% of the cases. Therapeutic approaches at treatment of BCC include surgical treatment, curettage, radiotherapy, laser treatment, 5-fluorouracil, interferon alpha (IFN), Photodynamic therapy (Lyubomir, *et al.*2013).

2.2.3.3 Melanoma:

The incidence of melanoma in white populations worldwide is increasing, especially in light skinned people with sun exposure. Melanoma is rare in populations with pigmented skin such as Asians, Africans (Claus, *et al.*2008).

Malignant melanoma is an aggressive, therapy resistant malignancy of melanocytes. Exposure to UV radiation, fair skin, dysplastic nevi syndrome and a family history of melanoma are major risk factors for melanoma development. Biopsies of primary tumor and sampling of draining lymph nodes are required for optimal diagnosis and staging. Therapy for early disease is predominantly surgical with a minor benefit with the use of adjuvant therapy (Markovic, *et al.*2007).

2.3 Risk factors for skin cancer:

2.3.1 Ultraviolet Radiation:

Over exposure to ultraviolet (UV) radiation may cause genetic changes (mutations) in skin cells. The genetic changes cause the affected cells to alter their behavior and may result in cancer. UV radiation also induces the production of very reactive chemicals, oxidants in affected cells which may cause some of the changes associated with aging and increases one's risk of developing cancer (Miller and Mihm, 2006).

2.3.2 Skin color:

Fair skin is more susceptible to UV radiation damage. Caucasian, specifically individuals with freckles, light eyes and/or red hair are at higher risk of skin damage that may lead to skin cancer (Rager, *et al.*2005).

2.3.3 Radiation therapy:

Radiation therapy is often used as a treatment of primary cancers. Irradiation of primary tumor often exposes normal skin to increased levels of radiation and may lead to the development of secondary cancers, including skin cancer. The cancer arises due to mutation within skin cells caused by high doses of radiation (Ron, 2003).

2.3.4 Chemical exposure:

Chronic exposure to arsenic, industrial tar and paraffin may increase one's risk of developing skin cancer (Holcomb, 2006).

2.3.5 Personal history:

Survivors of skin cancer have an increased risk of developing a secondary cancer. The greatest risk for relapse is within the first five years following treatment. The highest risk may be a result of a biological predisposition, previous exposure to skin cancer risks or a consequence of the initial cancer treatment itself (Miller and Mihm, 2006).

2.3.6 Immunosuppression:

Immunosuppression weakens the action and efficacy of the immune system and hinders its ability to fight foreign invasion. A weakened immune system is less able to eliminate cells that have suffered mutations and have the potential to develop into cancer cells (Lopez, *et al.*2006).

2.3.7 Infection:

Cutaneous human papilloma virus (HPV) infection may be a risk factor for SCC of the skin (Lannacone, *et al.*2012).

2.4 Diagnosis of skin cancer:

2.4.1 Skin biopsies:

Skin biopsy is a common dermatologic procedure that is typically required to assess cutaneous neoplasm and to evaluate indistinct skin eruptions for which a clinical differential diagnosis is considered (Sleiman, *et al.*2013).

The most common techniques include the punch, shave, excisional and incisional biopsies. The choice of different skin biopsies is dependent on the suspected diagnosis of the skin lesion (Achar, 1996).

2.4.2 Imaging techniques:

Various anatomical imaging techniques have been used to evaluate different types of skin cancer lesions, staging, preoperative planning and post treatment assessment including laser scanning confocal microscopy, optical coherence tomography, high-frequency ultrasound, terahertz pulsed imaging, magnetic resonance imaging, and photo acoustic microscopy (HaoHong, *et al.*2008).

2.5 Tumor markers:

These are substances present in or produced by a tumor itself or produced by host in response to a tumor that can be used to differentiate a tumor from normal tissue or to determine the presence of a tumor based on measurements in blood or secretions. Tumor markers can be used for screening and early detection of common cancers, diagnostic confirmation, prognosis and prediction of therapeutic response and monitoring disease and recurrence (Sharma, 2009).

2.6 CD10:

CD10 is a 90-110 KD a cell membrane metalloproteinase at 3q21-27 which inactivates bioactive peptides, including bombesin. In epithelial cells, CD10 loss from methylation leads to increased cell migration, cell growth and cell survival, contributing to neoplastic development and progression (Papandreou and Nanus, 2010).

In tumors of the skin, CD10 is expressed in dermatofibroma, dermatofibrosarcoma protuberans and melanoma (Pham, *et al.*2006). CD10 has

been expressed with some frequency in xanthomatous neoplasms of the skin (Perna, *et al.*2005).

Hultgren and Dimaio (2007) suggested CD10 expression to be helpful in distinguishing between atypical fibroxanthomas (strong diffuse expression in 94% of cases) and SCC (weak and patchy expression in 50% of cases).

Hayam and Hayam (2007) suggested CD10 might be a useful immunohistochemical marker to differentiate between BCC and SCC. If tumor cells were CD10 positive, this would favor BCC over SCC. Absence of CD10 in all the SCC and in infiltrating BCC together with its overexpression in the surrounding stromal cells might confer invasive properties to such tumors.

Yada, *et al* (2004) found that strong expression of CD10 in tumor cells of BCC (86%), and found that the smaller the number of positive tumor cells, the larger the number of positive stromal cells, in particular in sclerosing BCCs.

2.7 Treatment of skin cancer:

The choice of treatment depends on the tumor's location, size, borders and growth rate. Treatment has three goals include complete eradication of the cancer and preservation or restoration of normal function and cosmesis. The standard treatment approaches are superficial ablative techniques (electrodessication and curettage and cryotherapy) used primarily for low risk tumors and full thickness techniques (Mohs micrographic surgery, excisional surgery and radiotherapy) used to treat high risk tumors. Removal of the entire tumor is essential to limit and prevent tumor recurrence (Martinez and Otley, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3. Materials and Methods:

3.1 Materials:

Archived tissue blocks of skin cancer were used in this study.

3.2 Methods:

3.2.1 Study design:

This was a retrospective descriptive case study aimed to demonstrate CD10 expression in skin cancer using immunohistochemical method.

3.2.2 Study samples:

Forty skin tissue blocks were obtained from tissues previously diagnosed as BCC and SCC collected from The National Health Laboratory. Patient's identification; age and sex were obtained from patients files.

3.2.3 Sample cutting:

Section of three microns thickness was obtained from each formalin fixed paraffin wax embedded tissue using rotary microtome.

3.2.4 Sample staining:

3.2.4.1 Immunohistochemical staining:

Paraffin sections were immunostained using modified Dako indirect technique. Sections were heated in oven and cleared in two changes of xylene for two minutes. Then, hydrated through descending concentration of ethanol (100%, 90%, 70%, 50%) and water two minutes for each. Afterthat, Ag retrieved by Dako PT link retrieval technique using Tris-EDTA buffer (PH 9.0) for 20 minutes, then treated with hydrogen peroxide solution 3% for fifteen minutes, then washed in phosphate buffer saline (PH 7.4) for five minutes and treated with Ultra V Block for eight minutes. Sections were treated with CD10 primary antibody for twenty minutes, rinsed in phosphate

buffer saline, then treated with secondary polymer conjugate for thirty minutes, rinsed in phosphate buffer saline, treated with substrate and DAB chromogen for seven minutes, washed in phosphate buffer saline for 5-10 minutes, counterstained in Mayer's haematoxylin for one minute, washed and blued in running tap water for ten minutes, dehydrated through ascending concentration of ethanol (50%, 70%, 90%,100%) then cleared in xylene and mounted in DPX mounting media.

3.2.5 Result interpretation:

All quality control measures were adopted, and negative control section was performed by omitting the primary antibody during immunohistochemical staining.

Positive CD10 staining was identified as brown cytoplasmic staining with or without cell membrane staining.

3.2.6 Statistical analysis:

Data were analyzed computerized by using SPSS program version 11.5 frequencies, means and chi-square test were calculated.

3.2.7 Ethical consideration:

Samples were collected after acceptance from National Health Laboratory Histopathology Department administration for archive samples and patients data collection.

Chapter Four

Results

A total of 40 sample of patients diagnosed as skin cancer were used in this study. 12 (30%) of which were BCC, 28 (70%) were SCC as indicated in table (4.1).

The age of study population ranged between 17 and 95 years with mean of age 59.9 years. The study populations were grouped in two age groups, equal or younger than 50 years were 12 (30%) patients and older than 50 years were 28 (70%) patients as indicated in table (4.2).

The description of gender showed that 18 (45%) patients were males and 22 (55%) patients were females as indicated in table (4.3).

The description of tumor grade of SCC revealed that well differentiated tumor in 17\28 (60.7%) samples, moderate differentiated tumor in 8\28 (28.6%) samples and poor differentiated tumor in 3\28 (10.7%) samples as indicated in table (4.4).

The positive rate of CD10 within tumor cells was 8\12 (67%) among BCC and 2\28 (7.1%) among SCC and the negative rate of CD10 within tumor cells was 4\12 (33%) among BCC and 26\28 (92.9%) among SCC. This results show significant statistical association (P .value=0.000), as indicated in table (4.5).

The positive rate of CD10 within stroma was 1\12 (8.3%) among BCC and 17\28 (60.7%) among SCC, and the negative rate of CD10 within stroma was 11\12 (91.7%) among BCC and 11\28 (39.3%) among SCC. This results show significant statistical association (P . value= 0.002) as indicated in table (4.6). So there is a relation between histopathological diagnosis and CD10 expression within stroma.

Positive expression of CD10 within stroma is common among well differentiated squamous cell carcinoma in 13/17 (76.4%) samples and in 3/8 (37.5%) samples of moderate differentiated SCC with no expression in poor differentiated SCC. So there is a significant relation between CD10 expression and tumor grade (P.value=0.02), as indicated in table (4.7).

Table (4.1) Distribution of histopathological diagnosis among the study population:

Histopathological diagnosis	Frequency	Percent
Basal cell carcinoma	12	30
Squamous cell carcinoma	28	70
Total	40	100

Table (4.2) Distribution of age groups among the study population:

Age groups	Frequency	Percent
≤ 50	12	30
> 50	28	70
Total	40	100

Table (4.3) Distribution of gender among the study population:

Gender	Frequency	Percent
Males	18	45
Females	22	55
Total	40	100

Table (4.4) Distribution of tumor grade among squamous cell carcinoma:

Tumor grade of SCC	Frequency	Percent
Well differentiated	17	60.7
Moderate differentiated	8	28.6
Poor differentiated	3	10.7
Total	28	100

Table (4.5) Relation between histopathological diagnosis and CD10 expression:

CD10 expression	BCC	SCC	Total
Positive	8	2	10
Negative	4	26	30
Total	12	28	40

P.value=0.000

Table (4.6) Relation between histopathological diagnosis and CD10 expression within stroma:

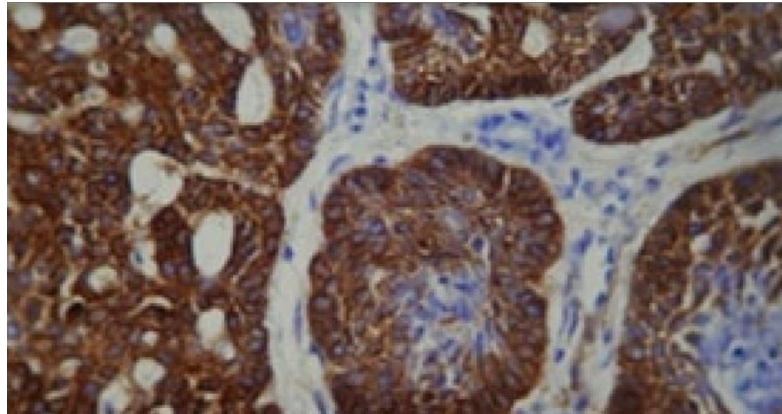
CD10 expression	BCC	SCC
Positive	1	17
Negative	11	11
Total	12	28

P.value=0.002

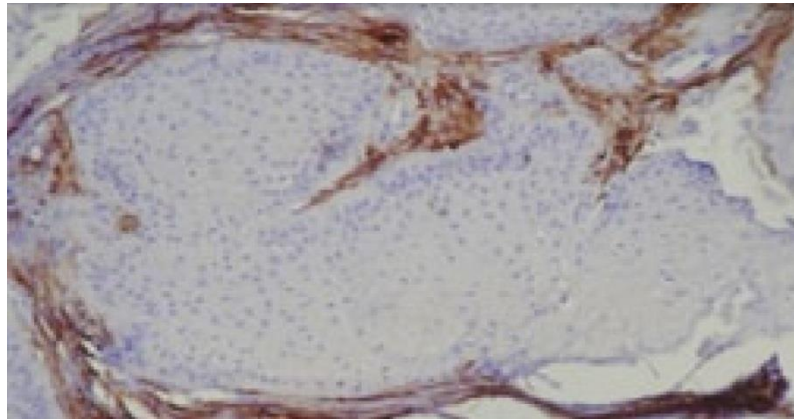
Table (4.7) Relation between CD10 expression within stroma and tumor grade of SCC:

CD10 expression	Tumor grade of SCC			Total
	Well differentiad	Moderate differentiaed	Poor differentiatd	
Positive	13	3	0	16
Negative	4	5	3	12
Total	17	8	3	28

P.value= 0.02



Micrograph (4.8) section of skin cancer stained using immunohistochemical modified Dako indirect technique for CD10 detection show positive CD10 expression within tumor cells in basal cell carcinoma (BCC).



Micrograph (4.9) section of skin cancer stained using immunohistochemical modified Dako indirect technique for CD10 detection, show positive CD10 expression within stromal cells in squamous cell carcinoma (SCC).

CHAPTER FIVE

DISCUSSION

Melanoma and non-melanoma skin cancers (NMSC) are now the most common types of cancer in white populations. Both tumor entities show an increasing incidence rate worldwide but a stable or decreasing mortality rate (Leiter, *etal.*2014). In Asians and Africans skin cancer is less common; the low incidence of skin cancer in dark skinned people is attributed to the increased epidermal melanin which provides photo-protection from UV radiation.

Sex distribution revealed that (45%) were males and (55%) were females. Patient age ranging between 17 and 95 years, with mean age 59.9 years. A similar results was observed by Ali (2014) his study reported that the mean of age of patients was 62.0 years and 57.5 years for squamous cell carcinoma and basal cell carcinoma respectively.

This study represent that the majority of skin cancer is SCC representing (67.5%). This result is similar to result observed by Abdelsamei, *et al* (2012) who reported that squamous cell carcinoma is the commonest skin malignancy in Sudan accounting for 42.6%.

The positive rate of CD10 within tumor cells was 8\12 (67%) among BCC and 2\28 (7.1%) among SCC, this results showed significant statistical association (P.value=0.000). This results are in accordance with another studies by Mitra, *et al* (2011) who repored that the expression of CD10 was noted in tumoral cells in 26/30 (86.7%) of BCC cases versus 1/26 (3.8%) of SCC cases (P<0.001). And another study by Shafaei, *etal* (2015) who reported that CD10 positivity within tumor cells in 25/42 (59.5%) of BCC cases with no expression in all SCC cases. And another study by Pourabdollah, *et al* (2014) who reported that the rate of CD10 expression in tumor cells was significantly higher in BCC 20/27 cases versus 2/17 cases of

SCC ($P < 0.0001$). Our results reflect the fact that BCC are biologically different from SCC.

The positive rate of CD10 within stroma was 1/12 (8.3%) among BCC and 17/28 (60.7%) among SCC. This results showed significant statistical association (P -value= 0.002) between stromal cells of BCC, SCC and expression of CD10. Our results are in accordance with another study by Mohammed and Rehab-Allah (2015) that reported stromal CD10 expression was positive in 75% of SCC cases. And supported with another studies by Yada, *et al* (2004) that reported CD10 positivity within stroma in all cases of SCC and supported by Fatemeh, *et al* (2013) that reported (100%) of SCC cases had stromal CD10 reactivity, comparison of CD10 expression between the BCC and SCC groups showed a significant difference ($P < 0.001$) in each of the tumor and stromal cells.

In our study, positive expression of CD10 within stroma in SCC is common among well differentiated squamous cell carcinoma in 13/17 (76.4%) cases and in 3/8 (37.5%) cases of moderate differentiated SCC with no expression in poor differentiated SCC. This result showed a significant relation between CD10 expression and tumor grade (P -value=0.02). Our results were not completely in accordance with previous study by Hayam and Hayam (2007) that reported CD10 was positive in 3/4 cases (75%) of well differentiated SCC and in 7/9 cases (78%) of moderately differentiated SCC and in all the 3 cases (100%) of poor differentiated SCC. Probably more studies must be done with more samples to exclude one of these findings.

The over expression of CD10 in the stromal cells of SCC and some variants of BCC suggest the invasive properties of such tumors. It was postulated that due to structural similarities of CD10 to matrix metalloproteinases (MMPs), CD10 could create a microenvironment that facilitates cancer cell invasion and metastasis (Talvensaaari, *et al*.1998). Increased stromal expression of CD10 had been related to tumor progression and metastasis in different

tumors. For example, inoral cavity SCC, CD10 stromal positivity was correlated to presence of metastasis, local recurrence and high tumor grade (Piatelli, *et al.*2006).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion:

On the bases of this study we conclude that:

CD10 can be utilized as a differential immunohistochemical marker to distinguish SCC from BCC. CD10 positivity in tumoral cells supports BCC as final diagnosis and at least SCC can be ruled out.

The expression of CD10 in the stromal cells of SCC and some of BCCs suggest the invasive properties of such tumors.

6.2 Recommendations:

On the basis of this study we recommend that:

1-Further studies with large sample size and with different histological subtype of BCCs and different grades of SCCs should be done.

2-Application of CD10 as differential immunohistochemical marker to distinguish between BCC and SCC in Histopathology laboratory.

CHAPTER SEVEN

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Appendices

Instruments and materials:

Instruments:

Rotary microtome

Oven

Coplin Jar

Staining racks

Glass slides

Cover glass

Water bath

Dako pen

Dako PT link

Materials:

Xylene

Ethyl alcohol (absolute, 90%, 70%, 50%)

Mayer's Haematoxylin

Distill water

Tris EDTA buffer

Phosphate buffer

Peroxidase blocker

Anti CD10 antibodies (primary antibodies) Clone 56C6.

Tris-EDTA buffer (PH 9.0) component:

10ml of 1M Tris-Cl buffer and 2ml of 0.5M EDTA solution.

Mix the solution with DW and make up the volume to 1000 ml.

Phosphate (PH 7.4) component:

Solution A (0.2 M sodium di-hydrogen orthophosphate, 3.12g di sodium hydrogen orthophosphate, 100 ml DW).

Solution B (0.2 M sodium di-hydrogen orthophosphate, 3.83g di sodium hydrogen orthophosphate, 100 ml DW) (9.5 ml from solution A + 40.5 ml from solution B).

Mayer's haematoxylin component:

Haematoxylin powder	1 gm
Potassium alum or ammonium alum	50 gm
Sodium iodate	0.2 gm
Citric acid	1 gm
Chloral hydrate	50 gm
Distilled water	1000 ml